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Metabolite analysis of *Clostridium acetobutylicum*: Fermentation in a microbial fuel cell

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ABSTRACT

Microbial fuel cells (MFCs) were used to monitor metabolism changes in *Clostridium acetobutylicum* fermentations. When MFCs were inoculated with *C. acetobutylicum*, they generated a unique voltage output pattern where two distinct voltage peaks occurred over a weeklong period. This result was markedly different to previously studied organisms which usually generate one sustained voltage peak. Analysis of the fermentation products indicated that the dual voltage peaks correlated with glucose metabolism. The first voltage peak correlated with acidogenic metabolism (acetate and butyrate production) and the second peak with solventogenic metabolism (acetone and butanol production). This demonstrates that MFCs can be applied as a novel tool to monitor the shift from acid production to solvent production in *C. acetobutylicum*.

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1. Introduction

MFCs present an unprecedented opportunity to reclaim energy directly from organic waste. The power density of an MFC is low compared to chemical or enzymatic fuel cells and the energy efficiency has been reported to range from 2% to 50% (Logan, 2008); however, MFC power is generated through natural metabolic processes while degrading a wide range of organic matter. Furthermore, based on the selection of organisms these cells produce traditional fuel chemicals as by-products of fermentation such as hydrogen, methane, and short chain alcohols, which can in turn power a conventional generation system. Since MFCs can simultaneously process waste and generate power, they can potentially increase the efficiency of municipal, manufacturing, or military systems while preventing environmental contamination from untreated waste (Logan, 2008; An et al., 2009).

The obligate anaerobe *Clostridium acetobutylicum* was used in industrial fermentations to produce the commodity chemicals acetone, butanol, and ethanol until the early part of the 20th century (Jones and Woods, 1986). The search for renewable fuel sources has revived interest in this organism since it can ferment complex carbohydrate sources found in many agricultural and industrial wastes (Qureshi et al., 2006). In batch fed fermentations *C. acetobutylicum* exhibits biphasic metabolism where during the initial acidogenic growth phase the cells produce high levels of butyrate and acetate. An accumulation of fermentation products and a drop culture pH causes the cells to enter the solventogenic growth phase where butyrate and acetate are converted to butanol and acetone, respectively (Jones and Woods, 1986). Previous studies have shown that *C. acetobutylicum* reduces artificial redox mediators and that this process alters the metabolic fluxes (Peguin and Soucaille, 1996). Additionally, we have demonstrated that MFCs inoculated with *Clostridium cellulolyticum* require artificial redox mediators for current production and these mediators alter metabolism (Sund et al., 2007). While it has been reported that *C. acetobutylicum* can be used in an MFC with the redox mediators methlyene blue and resazurin (Mathuriya and Sharma, 2009), here we demonstrate that *C. acetobutylicum* can generate current in MFCs without the addition of redox mediators and current output of MFCs can be used to monitor acidogenic and solventogenic metabolism. The exact mechanism of electron transfer by *C. acetobutylicum* is not known but the time constant of the current generation of the system is very different from that reported earlier, where a simple discharge was recorded for multiple common MFC redox mediators (Sund et al., 2007). Other researchers have shown that MFCs can be used to measure metabolic output (Biffinger et al., 2008; Favre et al., 2009), however this is the first instance of MFC use for sensing changes in an organism's metabolic pathway.

2. Methods

2.1. Reagents and biological materials

Culture growth and MFC operation, both in the anode and cathode chambers, was achieved with Clostridial growth medium

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14. ABSTRACT

Microbial fuel cells (MFCs) were used to monitor metabolism changes in Clostridium acetobutylicum fermentations. When MFCs were inoculated with C. acetobutylicum, they generated a unique voltage output pattern where two distinct voltage peaks occurred over a weeklong period. This result was markedly different to previously studied organisms which usually generate one sustained voltage peak. Analysis of the fermentation products indicated that the dual voltage peaks correlated with glucose metabolism. The first voltage peak correlated with acidogenic metabolism (acetate and butyrate production) and the second peak with solventogenic metabolism (acetone and butanol production). This demonstrates that MFCs can be applied as a novel tool to monitor the shift from acid production to solvent production in C. acetobutylicum.

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(CGM) based on the recipe previously developed by Wiesenborn et al. (1988). A 500 mL solution of CGM contained 25 g glucose, 2.5 g yeast extract, 1 g asparagine, 1 g $(NH_4)_2SO_4$, 0.5 g NaCl, 0.174 g $MgSO_4$, 5 mg $FeSO_4{\cdot}7H_2O$, 5 mg $MnSO_4{\cdot}H_2O$, 0.375 g K_2HPO_4, 0.491 g $KH_2PO_4{\cdot}3H_2O$, and 100 μL antifoam C emulsion. Cultures of *C. acetobutylicum* (ATCC824) were prepared from spore suspensions by 10 min of heat shock at 80 °C and overnight incubation in CGM under anaerobic conditions at 35 °C. The culture was then diluted with CGM by a ratio of 1:5 before inoculation into MFCs. All chemicals were purchased from Sigma–Aldrich (St. Louis, MO) and were molecular biology grade or the highest grade available.

2.2. MFC design/data collection

MFCs with graphite electrodes were constructed similarly to those described in the literature (Milliken and May, 2007) and as previously described by the authors (Sund et al., 2009). After assembly, each chamber was filled with 20 mL of CGM. The anode chambers were secured with rubber septa and the cathode chambers with a loose glass cap. Before inoculation, the electrodes, housings, and media were assembled and autoclaved. The MFCs were placed in a 35 °C incubator, linked to a 10 kΩ resistor, and allowed to discharge for a minimum of 120 min. The anode chambers of the MFCs were inoculated with 1 mL of the diluted *C. acetobutylicum* culture. The anode compartments were sealed with silicone septa and vented with 12-gauge syringe needles fed through a Millex-GP 0.2 μm sterile syringe filter unit (Millipore, Bedford, MA) through a length of plastic Tygon tubing into a flask of nitrogen-sparged water. This prevented buildup of metabolic gasses in the anode chamber while also preventing contamination of the chamber with O_2 or ambient microbes. The anode was entirely submerged in media and placed in direct contact with the lump graphite while the cathode was submerged just below the surface of the media in the cathode chamber. The potential across a 10 kΩ resistor was measured and recorded every 10 s via a DAQPad-6016 and a custom LabView® VI (National Instruments, Austin, TX).

2.3. Quantification of fermentation products

Fermentation products were quantified using a previously reported high performance liquid chromatography (HPLC) technique. (Ehrlich et al., 1981) At several points during the MFC run, 200 μL aliquots were withdrawn from the anode chambers, filtered through a Montage PCR centrifugal filter device (Millipore, Bedford, MA), and stored at −20 °C pending HPLC analysis. All MFC data was obtained in quadruplicate and fermentation was monitored by tracking pH and HPLC analysis.

The samples were analyzed by a 1200 series HPLC (Agilent Technologies) with a multi-wavelength detector (MWD) monitored at 250 nm and a refractive index detector (RID). An Aminex HPX-87H organic acid analysis column (Bio-Rad, Hercules, CA) heated to 30 °C was used for all separations. The samples were stored at 4 °C until ready for injection at volumes of 20 μL each. Each HPLC signal was analyzed for the presence of peaks corresponding to glucose, acetate, ethanol, butyrate, and butanol. These data were quantified using standardized concentration gradients (data not shown) on the HPLC column using HPLC grade reagents (Sigma–Aldrich, St. Louis, MO).

3. Results and discussion

Batch fed MFCs inoculated with *C. acetobutylicum* were monitored by measuring current as a voltage across a 10 kΩ resistor. There were two current peaks which occurred over a period of approximately 7 days. The initial current peak occurred approximately 40 h after inoculation and the second peak occurred approximately 150 h after inoculation (Fig. 1). In previous experiments other organisms exhibited a single peak and decay (Crittenden et al., 2006; Sund et al., 2007; Sund et al., 2009).

HPLC quantification of the metabolic products of *C. acetobutylicum* in the MFCs showed a close correlation between concentration of metabolites and current output (Fig. 1). A few hours after the flow of current started, butyrate and acetate concentrations rose sharply while butanol concentration remained close to zero. After the initial peak, current output slowly decreased and butyrate concentration leveled while butanol concentration began to rise rapidly. The trend was similar for acetate and ethanol although acetate concentrations leveled rather than decreased. The data shows that the concentrations of butyrate, acetate, ethanol, and butanol match their expected trends, with the acid generation occurring most prevalently during the first voltage peak and alcohol generation occurring noticeably in the second voltage peak. The concentration trends match the shift in metabolism and utilization of butyrate.

Fig. 2 shows that a rapid drop in the medium's glucose concentration occurs during the first measured voltage peak and slows during the intermediate period; this suggests the metabolism is slower due to excess butyrate decoupling the extracellular proton gradient. There is another rapid glucose consumption phase during the second measured voltage peak. The rate of glucose consumption was the most rapid when the fuel cell was producing the most current indicating that the two processes are coupled. Butyrate production rate was the highest during the first current peak while butyrate/butanol production rates were highest during the second current peak, (Figs. 1 and 2 summarized in Table 1) demonstrating that current output correlates with metabolic activity. During the initial current peak, butyrate and acetate concentrations rose sharply while the concentration of butanol remained close to zero indicating that the cells were in the acidogenic growth phase. Butanol concentrations rose dramatically in the intermediate period between the two peaks indicating that the culture was in the solventogenic growth phase.

We see further support for the metabolic shift in Fig. 2 where the pH drops through the initial voltage peak and increases in the time frame when butyrate and acetate would be utilized for

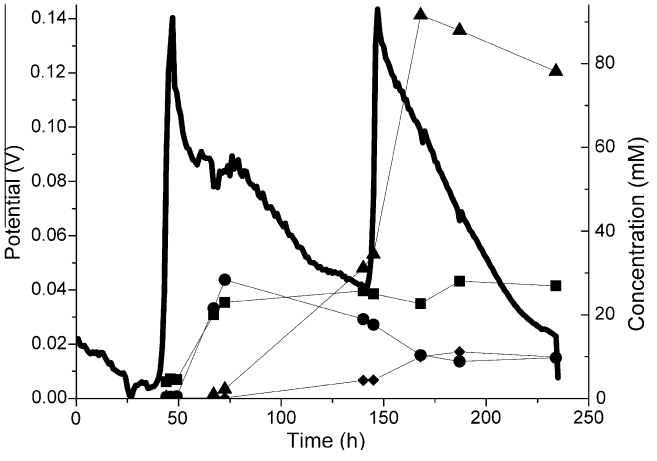

Fig. 1. Plot showing the time course of the potential (-Solid Line-) measured across a 10 kΩ resistor for a representative MFC containing *C. acetobutylicum* in the anode chamber. Note the two peaks generated by *C. acetobutylicum*. The plot also includes the concentrations of acetate (-Square-), butyrate (-Circle-), ethanol (-Diamond-), and butanol (-Triangle-) in the anolyte of this MFC through the time course of the experiment.

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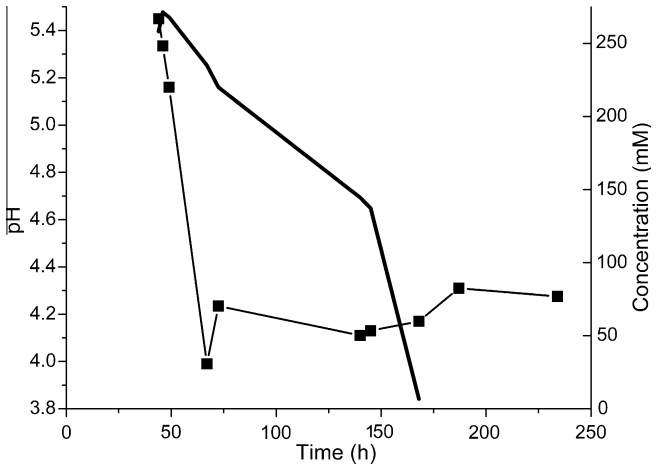

Fig. 2. Plot showing the time course of glucose utilization (-Solid Line-) and pH (-Square-) during the *C. acetobutylicum* fermentation in an MFC. Note that the concentration of glucose is from a representative MFC while the pH is an average of a total of six fuel cells (three trials of two fuel cells in each trial).

Table 1
Glucose utilization and production of butyrate and butanol (mM/h) in the MFC anolyte, calculated from chromatography data. Measureable quantities of butyrate started at about 50 h and butanol at about 67 h. Combined butyrate/butanol production is indicative of overall butyrate production.

Time (h)	Glucose utilization (mM/h)	Butyrate production (mM/h)	Butanol production (mM/h)	Butyrate + butanol production (mM/h)
46–49	1.1	–	–	–
49–67	1.8	1.2	–	1.2
67–72.5	2.7	1.2	0.2	1.5
72.5–140	1.1	−0.1	0.4	0.3
140–145	1.5	−0.3	0.7	0.4
145–168	5.7	−0.3	2.5	2.2
168–187	0.4	−0.1	−0.2	−0.3
187–234	0.0	0.0	−0.2	−0.2

solvent production. The open circuit voltage (OCV) of four independent MFCs are shown in the supplemental materials. OCV data also exhibits two peaks, although they are less pronounced than those measured by passing the generated current though a resistive load. The two peaks are of a similar timeframe as Fig. 1, with some smoothing due to averaging, indicating that this phenomenon is still linked to the culture's metabolism. The OCV exhibits significantly higher magnitude, 0.65 V which is approximately 0.2 V higher than previously reported studies with *Shewanella oneidensis* in identical hardware (Sund et al., 2009). It is suspected that this is due to the high redox potential of *C. acetobutylicum* ferredoxins involved in the process of converting pyruvate to acetyl-CoA. (Guerrini et al., 2008) This shift in E_0 is most likely the cause of the OCV difference between the two genera.

The data presented agrees with previous reports that initial fermentation of glucose by *C. acetobutylicum* is acidogenic where glucose is oxidized to lactate, butyrate, and acetate (White, 2007). Butyrate is lipophilic so it migrates from the outside of the cell to the cytosol (Monot et al., 1984); this eventually causes a disturbance in the proton gradient and impedes metabolism. The organism compensates for this decreased extracellular pH by shifting metabolism to the solventogenic phase and is capable of converting the acetate to acetone and butyrate to butanol thus moderating the pH. In nature these phases are capable of oscillating, but in the closed system of a batch fed fuel cell we did not see the shift back to the acidogenic phase under these experimental conditions. Fig. 1 shows this process where there is a lag in the metabolism during the shift from acidogenic to solventogenic phases, correlating with a lag in the current generation.

4. Conclusions

The data presented a direct correlation between voltage output and metabolic phase. This correlation between electrical output and solvent formation makes *C. acetobutylicum* an ideal candidate for studying biofuel production and cellular metabolism. In addition to the current generated by the MFC itself, the organism also produces H_2 gas, which may then power a conventional hydrogen fuel cell. The acetone, butanol, and ethanol generated as by-products of fermentation may be separated and used as biofuels or industrial solvents. By consolidating the functions of waste management, renewable power generation, and solvent production, *C. acetobutylicum* fuel cells have the potential to reduce organic wastes and increase opportunities to convert those wastes to usable energy.

This communication demonstrates that the metabolic activity of *C. acetobutylicum* can be tracked via current generation in an MFC. Current generation has proven to be reproducible and repeatable and was validated through measurements of pH, glucose consumption, and metabolite generation. This phenomenon could be utilized to design an inexpensive autonomous system consisting of graphite electrodes, an electrical load and a recordable voltmeter to track *C. acetobutylicum* fermentations. With MFC-based monitoring, time and resources would be saved by reducing the need for processing multiple HPLC samples and the need to rely on pH probe calibration over extended periods of time in complex matrices would be negated. In the near term, these fuel cells could be utilized as a research tool for metabolic studies where the current response of microbial fuel cells would be extremely useful. With continued development, future monitoring systems for bioproduction become possible.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.biortech.2010.06.149.

References

An, J., Kim, D., Chun, Y., Lee, S.-J., Ng, H.Y., Chang, I.S., 2009. Floating-type microbial fuel cell (FT-MFC) for treating organic-contaminated water. Environ. Sci. Technol. 43, 1642–1647.

Biffinger, J.C., Byrd, J.N., Dudley, B.L., Ringeisen, B.R., 2008. Oxygen exposure promotes fuel diversity for *Shewanella oneidensis* microbial fuel cells. Biosens. Bioelectron. 23, 820–826.

Crittenden, S.R., Sund, C.J., Sumner, J.J., 2006. Mediating electron transfer from bacteria to gold via a self-assembled monolayer. Langmuir 22 (23), 9473–9476.

Ehrlich, G.G., Goerlitz, D.F., Bourell, J.H., Eisen, G.V., Godsy, E.M., 1981. Liquid chromatographic procedure for fermentation product analysis in the identification of anaerobic bacteria. Appl. Environ. Microbiol. 42, 878–885.

Favre, M.-F., Carrard, D., Ducommun, R., Fischer, F., 2009. Online monitoring of yeast cultivation using a fuel-cell-type activity sensor. J. Ind. Microbiol. Biotechnol. 36, 1307–1314.

Guerrini, O., Burlat, B., Léger, C., Guigliarelli, B., Soucaille, P., Girbal, L., 2008. Characterization of two 2[4Fe4S] ferredoxins from *Clostridium acetobutylicum*. Current Microbiology 56, 261–267.

Jones, D.T., Woods, D.R., 1986. Acetone–butanol fermentation revisited. Microbiol. Rev. 50, 484–524.

Logan, B.E., 2008. Microbial Fuel Cells. John Wiley and Sons, Inc. Hoboken, New Jersey.

Mathuriya, A.S., Sharma, V.N., 2009. Bioelectricity production from paper industry waste using a microbial fuel cell by *Clostridium* species. J. Biochem. Tech. 1 (2), 49–52.

Milliken, C.E., May, H.D., 2007. Sustained generation of electricity by the spore-forming, Gram-positive, *Desulfitobacterium hafniense* strain DCB2. Appl. Microbiol. Biotechnol. 73, 1180–1189.

Monot, F., Engasser, J.-M., Petitdemange, H., 1984. Influence of pH and undissociated butyric acid on the production of acetone and butanol in batch cultures of *Clostridium acetobutylicum*. Appl. Microbiol. Biotechnol. 19, 422–426.

Peguin, S., Soucaille, P., 1996. Modulation of metabolism of *Clostridium acetobutylicum* grown in chemostat culture in a three-electrode potentiostatic system with methyl viologen as electron carrier. Biotechnol. Bioeng. 51, 342–348.

Qureshi, N., Li, X.L., Hughes, S., Saha, B.C., Cotta, M.A., 2006. Butanol production from corn fiber xylan using *Clostridium acetobutylicum*. Biotechnol. Prog. 22, 673–680.

Sund, C.J., McMasters, S., Crittenden, S.R., Harrell, L.E., Sumner, J.J., 2007. Effect of electron mediators on current generation and fermentation in a microbial fuel cell. Appl. Microbiol. Biotechnol. 76, 561–580.

Sund, C.J., Wong, M.S., Sumner, J.J., 2009. Mitigation of the effect of catholyte contamination in microbial fuel cells using a wicking air cathode. Biosens. Bioelectron. 24, 3144–3147.

White, D., 2007. The Physiology and Biochemistry of Prokaryotes, third ed. Oxford University Press, New York. pp. 383–403.

Wiesenborn, D.P., Papoutsakis, E.T., Rudolph, F.B., 1988. Thiolase from *Clostridium acetobutylicum* ATCC 824 and its role in the synthesis of acids and solvents. Appl. Environ. Microbiol. 54, 2717–2722.

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